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Beware of connective tissue proteins: assignment and implications of collagen absorptions in infrared spectra of human tissues

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Abstract

Infrared spectra of human central nervous system tissue and human breast carcinoma are presented. The spectra are discussed in terms of the composition of the tissues. It is shown that differences between spectra of white and grey matter can be rationalised on the basis of differences in lipid content. Spectra of the choroid plexus and arachnoid villus of the meninges show a series of absorptions not observed in other CNS tissue. These absorptions are discussed in terms of the connective tissue content of the samples. We demonstrate that the presence of collagen results in the appearance of a series of characteristic absorptions which may be mis-assigned as DNA phosphate absorptions. The implications of the presence of collagen in tissues for the diagnosis of disease states by IR spectroscopic methods, with particular reference to cancer, is discussed.

Keywords: Human tissue; FTIR; Collagen; Connective tissue; Cancer

1. Introduction

In the past two decades infrared spectroscopy has become an established tool for probing the structure and interactions of the complex materials (in particular, lipids, proteins and DNA) comprising biological tissues [1–3]. It is now routine for infrared spectroscopy to be applied to problems such as the determination of the secondary structure of proteins, conformational changes associated with ligand binding to proteins, phase behaviour and orientation of membrane components and the interconversion of DNA between the various physiologically relevant forms.

With advances in instrumentation, sampling and computational methods, it has recently become apparent that the wealth of knowledge obtained from studies of important biological materials in isolation or in simple mixtures can be transferred to the analysis of human tissues and the complex biochemical changes associated with pathological conditions. Preliminary studies have suggested that it is possible to use the sensitivity of IR spectroscopy to detect spectral changes which imply biochemical alterations on the molecular and supramolecular scale characteristic of various forms of cancer [4], multiple sclerosis [5], arthritis [6] and disorders of calcified tissue [7]. However, a complete understanding of the spectra of healthy and diseased tissues can only be achieved with a thorough knowledge of the composition and properties of tissues at the cellular and subcellular levels. In this paper we present IR spectra of various human tissues and discuss assignments of the major spectral features in light of their composition. We propose novel assignments for a series of absorptions between 1000–1400 cm^{-1} based upon spectra of the connective tissue protein collagen which may have important implications for our understanding of IR spectra of human tissues and the diagnosis of disease states based upon IR spectra.

2. Materials and methods

Human central nervous system tissue was obtained from locally performed autopsies or from the National Neurological Research Specimen Bank, VA Wadsworth Division

Abbreviations: CNS, central nervous system; FTIR, Fourier transform infrared.

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(Los Angeles, CA). Breast tissue was obtained from the Manitoba Breast Tumour Bank (Department of Pathology, University of Manitoba, Winnipeg, Manitoba). Type I collagen from calf skin was obtained from Sigma (St. Louis, MO).

Tissue samples were mounted either in a diamond anvil cell (High Pressure Diamond Optics, Tucson, AZ) or between a pair of CaF_2 windows with a 10 μm well etched on one face. Collagen was suspended in H_2O (50 mg/ml), 10 μl of the suspension evenly spread over the surface of a CaF_2 window and the sample allowed to dry to form an even film.

Spectra were recorded on Digilab 60 and 60A FTIR spectrometers equipped with liquid nitrogen cooled mercury cadmium telluride detectors and continually purged with dry air. For each sample 200 interferograms were collected, triangularly apodised and Fourier transformed to generate spectra with nominal resolution of 4 cm^{-1} (CNS tissue) or 2 cm^{-1} (breast tissue and collagen spectra). Residual water vapour was interactively subtracted. Fourier self-deconvolution was performed as previously described using software developed in our laboratory [8] with a resolution enhancement factor of 1.7 and a half bandwidth of 13.5.

3. Results

Representative infrared spectra of white matter (subcortical), grey matter (frontal cortex), choroid plexus and the arachnoid villus from the human central nervous system are shown in Fig. 1. These tissues differ substantially in structure and function. White matter contains the myelinated axons of neurons. Grey matter on the other hand contains predominantly non-myelinated neuronal cell bodies. The choroid plexus consists of a connective tissue matrix covered by cuboidal choroid plexus cells which secrete cerebrospinal fluid. Finally, the arachnoid villus is part of the membranous structure surrounding the brain (meninges) and is responsible for the re-absorption of cerebrospinal fluid. The presence of a substantial connective tissue matrix is the major difference between the choroid plexus and arachnoid villus and other areas of the central nervous system, in which mechanical support is provided by specialised cells rather than connective tissue.

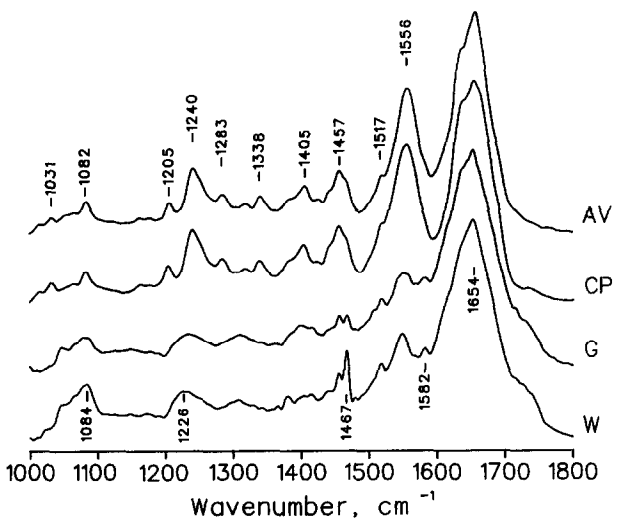

Fig. 1. Representative infrared spectra of white matter (W), grey matter (G), choroid plexus (CP) and the arachnoid villus of the meninges (AV).

The major feature in the spectra shown in Fig. 1, and in spectra of all tissues studied to date (with the exception of calcified tissues), is the amide I absorption band between 1610–1690 cm^{-1} which arises from the stretching vibration of the C=O groups of amide groups in proteins [2,3]. In isolated proteins and lipid-protein complexes the position of the amide I maxima is used to estimate protein secondary structure. However, as the amide I absorptions of all proteins present in tissue are observed simultaneously it is not usually possible to assign particular amide I absorptions to particular secondary structures within any one protein in IR spectra of tissues. Furthermore, significant contributions from non-protein components of tissues also exhibit absorptions in the amide I region of the spectrum. The most intense non-protein absorption in most tissues arises from the O-H bending vibration of water (a band at around 1640 cm^{-1}), and variations in the water content of tissues can cause significant variations in the amide I absorption profile. In addition, the amide groups of sphingolipids (particularly cerebrosides in the myelin of white matter) exhibit significant amide I absorptions. A number of amino acid side chain vibrations give rise to absorptions which overlap with the amide I band, in particular asparagine and glutamine. Finally, less intense absorptions also arise from the C=O groups of nucleotides in DNA and RNA. The complex nature of tissues therefore makes this spectral region (one of the most straight forward to interpret in isolated system) difficult to analyse and great care should be taken in any interpretation of spectral differences between tissues or in disease states.

The spectral region 1500–1600 cm^{-1} is almost as complex as the amide I region. The most intense absorption in this region is the amide II mode, generally observed at around 1550 cm^{-1}, which arises from the N-H bending vibration strongly coupled to the C-N stretching vibration of protein amide groups. In addition absorptions from the side chains of amino acids (arginine, aspartate, glutamate and tyrosine) and N-H groups of nucleotides are also seen.

Significant differences are apparent between spectra of the four types of tissue in the region 1500–1700 cm^{-1} which must represent differences in the composition of the tissue types. The most noticeable difference is the variation in the amide I/amide II intensity ratio. As discussed above, the O-H bending vibration of water gives rise to a strong absorption in the amide I region of the spectrum and it is the presence of this absorption that leads to the

differences in the amide I/amide II intensity ratio. Of the four tissues, grey matter contains the highest proportion of water (around 80%) and so has the largest contribution from the O-H bending vibration, leading to a significantly elevated amide I/amide II ratio. White matter has a somewhat lower water content (70%) and so the amide I/amide II ratio is reduced. The choroid plexus and arachnoid villus samples on the other hand are high in connective tissue and as a consequence have reduced water content. The reduced amide I/amide II ratio in these samples resembles that seen for isolated proteins, suggesting that protein C=O absorptions dominate this spectral region in such samples.

The increased relative intensity of the absorptions at 1517 and 1582 cm^{-1} suggests a greater contribution from tyrosine and arginine respectively in spectra of grey matter and white matter as compared to the choroid plexus and arachnoid villus. Again, this can be related to the composition of the tissues. The major connective tissue protein is collagen, which has a relatively low tyrosine and arginine content. Thus, the tyrosine and arginine side chain contributions in spectra of tissue with a significant connective tissue matrix are reduced.

The spectral region between 1000–1500 cm^{-1} is the most crowded in IR spectra of tissues, containing absorptions from many biologically important chromophores. Differences between the four tissues are again apparent. The major difference between spectra of grey matter and white matter is a reduction in the ratio of the absorption at 1467 cm^{-1} (CH_2 bending vibration) to that at 1456 cm^{-1} (CH_3 asymmetric bending vibration) in grey matter which reflects a decreased lipid content in grey matter. If we consider delipidation of white matter, it is apparent that for every CH_3 group we lose around 18 CH_2 groups are lost. Thus, the intensity of the CH_2 absorption falls much more rapidly than the intensity of the CH_3 absorption and we see the ratio of these two absorptions approaching unity in grey matter. In contrast, the spectra of the choroid plexus and arachnoid villus are dominated by the CH_3 asymmetric bending vibration in this region of the spectrum. A small increase in the intensity of the feature at approx. 1400 cm^{-1} in grey matter, choroid plexus and the arachnoid villus (attributed to the symmetric COO^- stretch of ionised fatty acids and amino acid side chains) suggests an increased contribution from carboxylate groups.

The remaining features are similar in spectra of grey and white matter and are assigned to the asymmetric (1226 cm^{-1}) and symmetric (1084 cm^{-1}) stretching vibrations of the PO_2^- groups of DNA, RNA and phospholipids, all of which show overlapping absorptions in this region of the spectrum. However, striking differences are seen between spectra of the choroid plexus and arachnoid villus and those of white and grey matter between 1000–1300 cm^{-1}. Prominent absorptions at 1283, 1240, 1205, 1082 and 1031 cm^{-1} in the spectrum of the choroid plexus and arachnoid villus are not present in spectra of healthy white or grey matter. It is reasonable to speculate that such absorptions are characteristic of the connective tissue present in the choroid plexus and arachnoid villus. In this respect it is interesting to note that a recent study on human cervical tissue reported spectra which have many features in common with our spectra of choroid plexus and meninges [9]. While it is therefore tempting to assign these unusual absorptions to connective tissue components, the question remains as to the precise nature of the chromophores giving rise to the major features.

Assignment of IR absorptions to precise constituents of connective tissue requires an understanding of the structure and function of this complex tissue. Connective tissue is ubiquitous but varies in composition throughout the body. It includes epithelial basement membranes (rich in type IV collagen), matrix (rich in type I and III collagen, proteoglycans and other fibrous proteins) and cellular components that include fibroblasts, blood vessels and adipocytes. The major connective tissue protein is collagen (about 30% of all body protein and 6% of total body mass). Collagen exists as a triple stranded helix, a structure favoured by the unique amino acid composition (25% proline/hydroxyproline, 33% glycine), stabilised by the formation of numerous interstrand hydrogen bonds and conferring remarkable mechanical properties to the protein. Hydroxylated amino acids interspersed along the helices serve as the attachment point for carbohydrate residues.

The IR spectrum of a film of type I collagen, the most abundant of the numerous types of collagen which exist, is shown in Fig. 2, together with the spectrum of a more typical protein (haemoglobin) for comparison. The protein was analysed as a film firstly because collagen has limited solubility in water at neutral pH and secondly because hydration of the insoluble collagen in the connective tissue matrix may be expected to be very low.

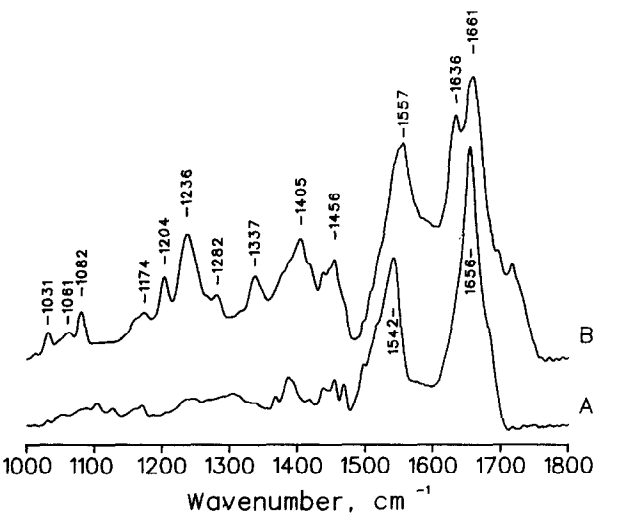

Fig. 2. Infrared spectra of films of haemoglobin (A) and type I collagen (B).

It is immediately apparent that the spectrum of collagen exhibits a number of absorptions which are not seen in the spectrum of haemoglobin or are greatly reduced in intensity. Of particular interest is the series of absorptions between 1000–1400 cm^{-1}, which show a striking resemblance to those seen in the spectra of the choroid plexus and the arachnoid villus. The features at 1031, 1061 and 1082 cm^{-1} occur in a region of the spectrum populated by C-O stretching absorptions and as such are assigned to C-O, stretching vibrations of the carbohydrate residues present in collagen but not in haemoglobin. The strong series of absorptions between 1200–1300 cm^{-1} are more difficult to explain. Absorptions in this spectral region are generally assigned to the asymmetric stretching vibration of the phosphate groups of DNA, RNA, phospholipids and phosphorylated proteins. Collagen however is not phosphorylated. In addition, if absorptions in this region of the spectrum arise from asymmetric stretching vibrations of phosphate groups then we would expect to see a strong symmetric counterpart at about 1086 cm^{-1}, which is clearly not present. We are therefore confident that these features do not arise from phosphate groups.

The unique amino acid sequence and/or structure of collagen must be responsible for the unusual spectroscopic characteristics of the protein. The high proportion of glycine and proline residues is the most noticeable difference between the sequence of collagen and other proteins (accounting for almost 60% of all amino acid residues in collagen) and it is reasonable to speculate that these two amino acids are in some way responsible for the spectral characteristics we see between 1200–1400 cm^{-1} in collagen. IR spectra of solid polyproline and polyglycine (not shown) show a number of absorptions in the region 1200–1400 cm^{-1} which have many similarities with absorptions in this region of the spectrum of collagen. Absorptions in this spectral region are generally attributed to the amide III, a complex vibrational mode having components due to C-N stretching and N-H in plane bending from amide linkages. In addition, significant absorptions arising from the wagging vibrations of CH_2 groups are also seen in this region of the spectrum. We therefore tentatively assign the discrete absorptions seen at 1204, 1236 and 1282 cm^{-1} in collagen to the amide III vibration with significant mixing with the CH_2 wagging vibration from the glycine backbone and proline side chains. The increased intensity of this series of absorption in collagen is difficult to explain. However, it may be related to the unusual nature of the side chains of glycine and proline, which may have an effect upon the intensity of the amide III due to mixing with the CH_2 wagging vibration. Furthermore, the amide II absorption shows some conformational sensitivity, and this conformational sensitivity may be expressed not only as frequency dependence but also as intensity dependence. Thus, the unusual structure of collagen may give rise to an unusually intense amide III absorption. Finally, the C-N stretching vibration of the cyclic proline side chain may also contribute intensity in this spectral region. An additional absorption seen in collagen and polypeptide films at 1337 cm^{-1} is attributed predominantly to the CH_2 wagging vibration of proline side chains.

Combining our results on brain tissue and protein films, we are now in a position to more confidently assign the series of absorptions between 1000–1350 cm^{-1} in the spectra of choroid plexus and arachnoid villus predominantly to vibrations associated with collagen carbohydrate residues (1000–1100 cm^{-1}) and the amide III/CH_2 wagging vibrations of the collagen (1200–1350 cm^{-1}).

4. Discussion

The assignment of features in the IR spectrum of human tissues to collagen has important consequences. As the most abundant and ubiquitously distributed protein in the human body, significant collagen absorptions may be expected in almost all tissues (most areas of the CNS and blood being the only exceptions). Failure to account for the presence of collagen in tissues will lead to incorrect assignments. Furthermore, the presence of collagen in tissues will have important implications for the diagnosis of disease states. The connective tissue matrix is considerably altered in a number of diseases and the nature of the alteration may depend upon the stage of the disease. For example, significant changes in the connective tissue matrix are associated with both age and a range of proliferative conditions of the breast. Benign fibrocystic disease involves ductal epithelial proliferation, cystic changes and, in may cases, a dominant collagenous component in the stroma. Benign epithelial tumours such as fibroadenoma are composed of both an epithelial component together with a substantial stromal neoplastic element with variable collagen deposition. In situ ductal carcinoma of the breast often stimulates a prominent pre-ductal stromal response and the transition to invasive carcinoma is similarly associated in many cases with a marked stromal fibroblastic proliferation and collagen deposition. Tumour progression is also associated with changes in the basement membrane, which is normally maintained and modeled by a balance of secreted products from both epithelial and adjacent stromal cells. Alteration of this balance and degradation of the basement membrane is a significant factor in the process of invasion by malignant tumour cells. Thus, the overall and regional stromal and collagenous composition of an organ such as the breast can vary significantly with pathological states.

It has been suggested that IR spectroscopy is a potentially useful tool for the diagnosis of cancer [4]. Considerable differences have been observed between IR spectra of normal and malignant tissue. Of particular note are changes in absorptions attributed to the asymmetric stretching vibration of DNA phosphate groups. In normal tissue, an absorption at 1240 cm^{-1} has been attributed to the PO_2^-

asymmetric stretching vibration of phosphate groups in DNA which are not involved in hydrogen bonds. In malignant tissue a shift to lower wavenumbers is seen, which upon application of band narrowing routines (such as derivation) may be seen to arise from the appearance of an absorption at around 1225 cm^{-1}, attributed to the PO_2^- asymmetric stretch of phosphate groups of DNA involved in hydrogen bonds. It has been suggested that such spectral changes may indicate changes in the hydrogen bonding pattern of DNA [4] which in turn indicates structural alterations in the DNA of malignant cells.

Based upon the results presented here the spectral changes reported in malignant tissue may have an alternative explanation. As we have demonstrated, significant absorptions from collagen may arise in infrared spectra of human tissues which strongly overlap with absorptions arising from the phosphate groups of DNA, which in isolated DNA and nuclei have been shown to absorb at around 1225 cm^{-1} [4]. Thus, the feature centered at 1240 cm^{-1} in human tissue may actually be a composite absorption dominated by absorptions arising from collagen rather than DNA. As discussed, variations in the connective tissue, and so collagen content of malignant tissue are known to occur and such variations will have pronounced effects on the IR spectrum of malignant tissues. Decreases in the amount of collagen present in samples, consistent with a highly advanced, invasive carcinoma, will lead to a decrease in the intensity of the collagen absorptions seen at 1204, 1240 and 1283 cm^{-1}. Malignant cells are characterised by genetic changes that are reflected in structural nuclear changes such as altered chromosomal structure or DNA content and functional changes in the control of cell division and growth. Increased DNA content (aneuploidy) will increase the intensity of the PO_2^- asymmetric stretching absorption of DNA. These two changes (decreased intensity at 1240 cm^{-1} due to collagen degradation and increased intensity at 1225 cm^{-1} due to elevated DNA levels) will result in an apparent shift of the composite collagen/DNA absorption to lower wavenumber and the appearance in resolution enhanced spectra of a feature at 1225 cm^{-1}. Thus, rather than denoting a primary biochemical event in cell transformation (i.e., alterations in DNA structure) the spectroscopic changes may actually reflect a secondary event, the alteration of the connective tissue matrix in which the cells sit.

Our hypothesis is strengthened by examination of spectra of human breast tumours (Fig. 3). The tumour from which the spectrum in Fig. 3 was obtained was pathologically classified as invasive ductal carcinoma. As discussed above, previous studies [4] suggest that tissue with such a high proportion of malignant cells would give rise to a significant absorption in resolution enhanced spectra at around 1225 cm^{-1}. However, in this case it is evident that the major absorption between 1200–1300 cm^{-1} is seen at 1241 cm^{-1}. No significant feature could be detected at 1225 cm^{-1}. Histological assessment of sections immediately adjacent to those that were analysed demonstrated that approx. 60% of the cross sectional area was invasive epithelial cells and 40% was stroma, consisting mainly of collagen and fibroblasts. This is typical of the composition of breast tumours, and the relatively large connective tissue content gives rise to significant absorptions at 1204, 1241 and 1283 cm^{-1}, assignable to collagen amide III/CH_2 wagging and C-N stretching absorptions, which are of sufficient intensity to obscure the DNA PO_2^- asymmetric stretching absorption at 1225 cm^{-1}. Thus, it is not possible to describe changes in DNA structure and/or interactions based upon the infrared spectrum of the breast tumour described here due to interfering absorptions from collagen.

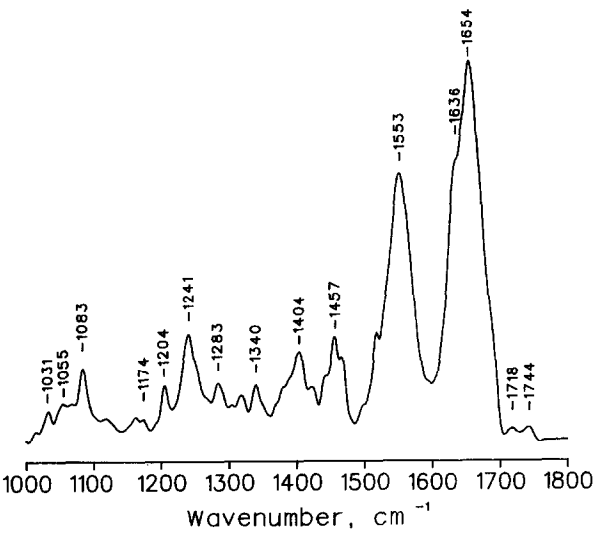

Fig. 3. Infrared spectrum of invasive ductal carcinoma of the human breast.

It should be stressed that the results presented here do not preclude the use of IR spectroscopy for the diagnosis of cancer. Rather they emphasise the care which must be exercised in obtaining pathologically defined tissue and the requirements for an extensive understanding of the nature of the tissues to be investigated.

In summary, we have presented IR spectra which highlight the problem associated with the presence of connective tissue in almost all samples of human tissues. Failure to appreciate the nature and extent of the connective tissue matrix in these samples, and the transformations in the matrix which may occur in disease, will almost certainly lead to errors in interpretation of spectra.

Acknowledgements

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